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14. ABSTRACT
Tuberous sclerosis complex (TSC) is an autosomal disorder resulting from mutations in the TSC1 or TSC2 genes that is associated with epilepsy, cognitive disability, and autism. TSC1/TSC2 gene mutations lead to developmental alterations in brain structure known as tubers in over 80% of TSC patients. Loss of TSC1 or TSC2 function in tubers results from biallelic TSC gene inactivation and leads to activation of the mTOR cascade as evidenced by phosphorylation of ribosomal S6 protein (P-S6). We demonstrate that there are numerous cytoarchitectural abnormalities in non-tuber brain areas in post-mortem TSC brain. Many of these regions exhibit aberrant phosphorylation of the ribosomal S6 protein (phospho-S6 or P-S6), a marker for enhanced mTOR signaling. We find P-S6 expression in cortex as well as subcortical regions including the cerebellum. Single cell mutational analysis of these regions reveals somatic missense mutations suggesting that even though these lesions are distinct from tubers, they arise by biallelic gene inactivation. We also identify a new marker protein, GFAP delta, that is co-expressed in tubers and subependymal giant cell tumors.

15. SUBJECT TERMS
tuberous sclerosis, mTOR, somatic mutation, ribosomal S6 protein

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Introduction

Tuberous sclerosis complex (TSC) is an autosomal disorder resulting from mutations in the TSC1 or TSC2 genes that is associated with epilepsy, cognitive disability, and autism. TSC1/TSC2 gene mutations lead to developmental alterations in brain structure known as tubers in over 80% of TSC patients. Loss of TSC1 or TSC2 function in tubers results from biallelic TSC gene inactivation and leads to activation of the mTOR cascade as evidenced by phosphorylation of ribosomal S6 protein (P-S6). Several new findings warrant further investigation of the mechanisms through which TSC gene mutations lead to developmental alterations in brain structure. Recent MRI studies suggest that there are subtle widespread abnormalities in TSC brains that contribute to neurocognitive deficits and *in vitro* evidence suggests that reduction of Tsc1 in rat neurons leads to altered dendrite structure.

First, we proposed to define subtle structural alterations distinct from tubers in post-mortem TSC brain specimens in the cortex, thalamus, basal ganglia, and cerebellum which may contribute to epilepsy, infantile spasms, and neurocognitive abnormalities in TSC using neuronal and astrocytic protein markers. Then, we hypothesized that P-S6 is expressed in these non-tuber brain lesions as well as tubers reflecting mTOR cascade activation similar to tubers. Next, we proposed to identify somatic second hit mutations in single microdissected P-S6 labeled cells in non-tuber brain lesions as a strategy to define whether all structural abnormalities in TSC require biallelic TSC gene inactivation. We have sought to determine whether P-S6 labeled giant cells in tubers and non-tuber brain lesions express a single or multiple somatic second hit mutations to test the hypothesis that structural lesions form by a clonal cellular expansion. Finally, we proposed to defined in an ongoing fashion new marker proteins that provide insights into lesion formation in TSC. During the two-year funding period, we have made strides in accomplishing all of the proposed goals. We have presented our work at national meetings, and have 2 papers submitted and one in preparation that summarizes our work.

Body

Clinical Features

The tuberous sclerosis complex (TSC) is an autosomal dominant disorder affecting children and adults resulting from mutations in one of two genes, *TSC1* (TSC1) or *TSC2* (TSC2) (ECTS, 1996; van Slegtenhorst et al., 1997). TSC is estimated to occur in 1:8000 live births (O'Callaghan et al., l998). TSC affects multiple body organ systems including the heart, kidney, skin and eye (Roach et al., 1998). However, the most disabling manifestations of TSC reflect abnormalities in brain function. For example, epilepsy occurs in over 70-80% of TSC patients and infantile spasms, a devastating epilepsy syndrome often associated with profound mental retardation and dismal neurological prognosis, occurs in 20-30% of babies with TSC (Sparagana and Roach, 2000). Comorbid neuropsychological disorders such as autism, mental retardation (MR), pervasive developmental disorder, attention deficit disorder (ADD), and obsessive-compulsive disorder (OCD) are common in TSC patients (Prather and de Vries, 2004). Thus, TSC is a common cause of significant and disabling neurological, cognitive, and behavioral disorders in children and adults.

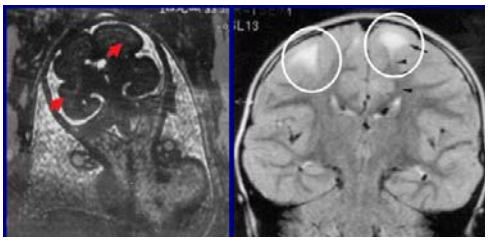

Figure 1. Left, Fetal brain MRI depicting two tubers at 25 weeks gestation (arrows). Right, two tubers in mature brain (circled).

Neuropathological Features

The neurological manifestations of TSC are believed to result from structural abnormalities in the brain that form as a consequence of TSC gene mutations. Tubers (Figs.1 and 2), present in over 80% of pediatric or adult TSC patients, are focal developmental abnormalities of cerebral cortical cytoarchitecture that are characterized histologically by disorganized cortical lamination and the presence of cells with aberrant morphologies such as dysplastic neurons (DNs), large astrocytes, and a unique cell type known as giant cells (GCs; Huttenlocher and Wollman, 1991; Crino and Henske, 1999). Tubers are single or multiple lesions detected by neuroimaging that form during embryogenesis. Tubers have been identified in fetal life as early as 20 weeks gestation (Fig.1). In older children and adults, tubers frequently calcify. Tubers are believed to be an important cause of epilepsy in TSC and for many patients who do not respond to AEDs, surgical resection of a tuber is necessary to achieve seizure control (Koh et al., 2000).

However, in the few reported neuropathological analyses of the post-mortem TSC brain, disruption of normal brain architecture distinct from tubers including small structural abnormalities including heterotopias, subcortical nodules, radial migration lines, areas of hypomyelination, and small cortical dysplasias have been described (Richardson, 1991). These lesions differ from tubers in that they are smaller, GCs are an infrequent finding, cortical lamination is mildly altered, and they do not exhibit calcification,

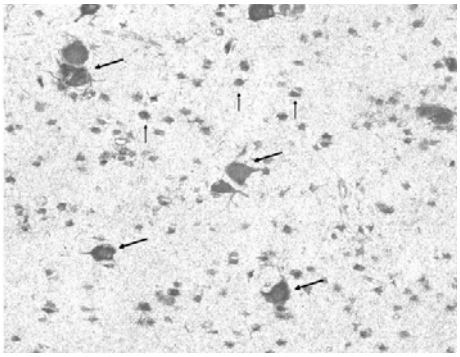

Figure 2. Tuber probed with MAP2 antibodies. GCs (large arrows) are distributed from the pial surface to the subcortical white matter without clear radial or laminar orientation and they may appear in clusters or lines. DNs are smaller (small arrows) and interspersed with GCs.

Recent MRI analyses in TSC patients have confirmed subtle structural abnormalities outside of tubers in the cortex and within subcortical structures such as the thalamus and basal ganglia (Ridler et al., 2001; Bolton et al., 2002) and suggest that these non-tuber brain lesions, in addition to tubers, may contribute to autism and cognitive disability in TSC. The histopathology of these lesions has not been comprehensively investigated and the mechanistic relationship of these abnormalities to TSC gene mutations is unknown i.e., do these lesions form by similar processes as tubers, are they secondary events, or are they a unique phenotype of TSC? In addition, while activation of the mTOR cascade is a robust finding in tubers, it is unclear whether mTOR activation occurs in non-tuber lesions. Moreover, a compelling observation is that some TSC patients exhibit profound neurological disorders i.e., infantile spasms or autism, but have **normal** neuroimaging studies. Likely, there are microscopic structural alterations not detectable by MRI that can disrupt neurological function. Thus, an important new perspective on neurological manifestations of TSC is to fully consider the effects of radiographically visible lesions (tubers) as well as radiographically minimal or occult lesions on brain function.

Recent functional neuroimaging studies have demonstrated altered [(18)F]fluorodeoxyglucose (FDG) and alpha-[(11)C]methyl-l-tryptophan (AMT) uptake (Eluvathingal et al., 2006) in the cerebellum of autistic TSC patients. A previous case study has shown that focal dysplasias can be identified in the cerebellum in TSC (Jay et al., 1998). In view of the proposed role for the cerebellum in normal cognition and possibly in autism, these findings suggest that that altered function within the cerebellum in TSC may be a new area for investigation.

mTOR Activation and Biallelic TSC Gene Inactivation

Mutations in *TSC1* or *TSC2* likely have a significant impact on neuroglial development (see Marcotte and Crino, 2005). TSC1 and TSC2 form a functional protein-protein heteromeric complex that constitutively inhibits the activation (phosphorylation) of mTOR (mammalian target of rapamycin), p70-S6-kinase, and ribosomal S6 proteins (Fig.3) that contribute to ribosomal assembly and protein translation (Arrazola et al., 2002; Kenerson et al., 2002). The mTOR flows downstream of the insulin-like growth factor-1 (IGF-1) receptors, PI3K, and Akt and serves as a key regulator of cell size via effects on ribosome biosynthesis and 5'-cap dependent mRNA translation (Schmelzle and Hall, 2000; McManus and Alessi, 2002). Constitutive negative modulation of this cascade by TSC1-TSC2 results in growth suppression, diminished protein synthesis, and restricted cell size. However, in response to growth factor stimulation e.g., IGF-1, nutrient availability, or stress, TSC2 is inactivated via Akt-mediated phosphorylation and causes Rheb (Ras homolog expressed in brain) mediated phosphorylation (activation) of mTOR, p70S6 kinase, ribosomal S6, and 4EIBP.

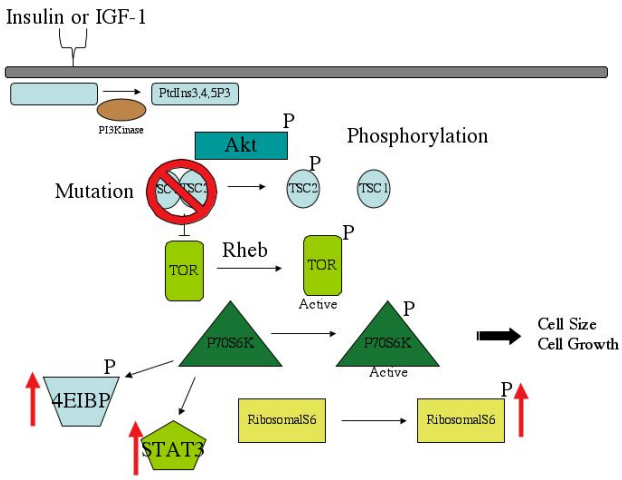

Figure 3. Activation of the mTOR cascade by TSC2 phosphorylation or TSC1/TSC2 mutation.

In TSC lesions, loss of TSC1 or TSC2 function leads to mTOR cascade activation and aberrant phosphorylation of ribosomal S6 protein (P-S6; Tee et al., 2002; Inoki et al., 2002). In keeping with the Knudsen "two-hit" mutational model, inactivation of both TSC1 or TSC2 alleles is necessary for mTOR activation and lesion formation (Green et al., 1994; Henske et al., 1999). By this mechanism, a

somatic “second hit” mutation superimposed on an existing germline mutation leads to loss of TSC1 or TSC2 function. Phosphorylation of ribosomal S6 protein is increased in subependymal giant cell tumor specimens from TSC patients (Chan et al., 2004) that exhibit biallelic inactivation. Recent work form our lab (Baybis et al., 2004) has demonstrated cell specific activation of the mTOR cascade in giant cells in human tubers as evidenced by P-S6 expression. Our lab was the first to demonstrate that expression of phospho-ribosomal S6 (P-S6) protein is a robust marker for cells lacking TSC1 or TSC2 function in tubers.

Key Research Accomplishments

The mission of our ongoing funding cycle based on the proposed Statement of Work has been to define how changes in brain structure result from alterations in TSC gene function. Over the past year we have optimized strategies for single cell microdissection, single cell gene mutation analysis, and morphometric analysis of post-mortem TSC brain tissue.

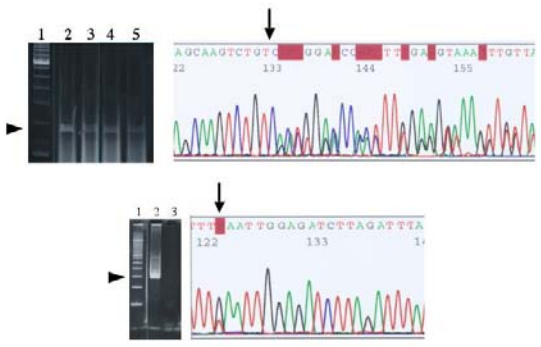

Figure 4. Mutation analysis in tubers. Top, germline deletion mutation (arrow) detected in all microdissected cells (genomic DNA). Bottom, nonsense “second hit” mutation (arrow) detected only in P-S6 labeled GCs.

Single Cell Gene Mutational Analysis

We have designed a strategy to sequence *TSC1/TSC2* in single microdissected P-S6 labeled cells. For example, in one tuber specimen, we identified a germline exon 15 *TSC1* mutation (delG2023) that was confirmed by clinical blood screening. P-S6 labeled GCs were microdissected using laser capture microdissection. All 23 exons of *TSC1* were sequenced in a pooled sample of 50 microdissected GCs. For comparison, 50 morphologically normal, non-PS6 labeled cells from the edge of the tuber resection were also microdissected. The germline exon *TSC1* mutation was detected in GCs and adjacent neurons in the tuber sample. A somatic (nonsense) *TSC1* mutation was detected in exon 22 (3096G>T, 959E>X) in P-S6 labeled GCs (Fig.4) but not DNs, morphologically normal neurons, or peripheral blood form the patient. Sequencing of *TSC2* did not reveal mutations in blood.

These experiments demonstrate for the first time the mutational mechanisms that lead to tuber formation and provide a novel strategy that can be applied to **defining the spectrum of germline and somatic second hit mutations in tubers** and non-tuber brain lesions. These results also allowed us to propose a model for tuber formation during brain development (Fig.5) in which a progenitor cell sustains a somatic “second hit” mutation early in corticogenesis (Fig.9, red cell), continues to divide, and generates progeny lacking functional *TSC1* or *TSC2* (Yu et al., in preparation). As a consequence, the mTOR cascade is activated, leading to cytomegaly and perhaps, impaired migration or lamination. Tubers form as a mosaic lesion of null cells containing germline and somatic TSC gene mutations (in red) and

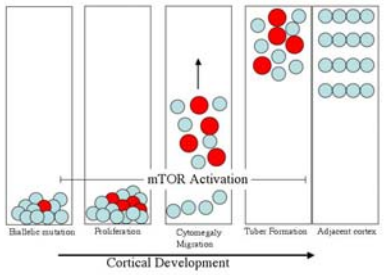

Figure 5. Two hit model of tuber formation during brain development.

haploinsufficient cells (e.g., dysplastic neurons, depicted in blue), containing only germline mutations. Interestingly, in this model the genotype of cells in adjacent non-tuber cortex (depicted in blue to the right of the tuber) is the same as dysplastic neurons (also in blue) within the tuber.

There are several unresolved issues relating to structural abnormalities and TSC1/TSC2 function in the developing brain. For example, an important unanswered question is whether tubers are formed by a single somatic mutation (as in Fig. 5) or by multiple second hit mutations (Fig.6, cells in red or green). A logical next question is whether somatic mutations occur at a developmental critical period and whether they occur simultaneously in a "shower" of mutational events. These notions have obvious importance for the realistic development of *in utero* therapy to prevent tuber formation (Crino, 2004).

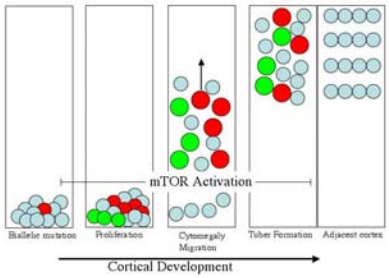

Figure 6. Multiple second hit mutations in distinct cells (green or red cells) leading to mTOR activation and tuber formation.

Selective Activation of mTOR Pathway in Non-Tuber Lesions

A new direction in understanding the broad picture of neurological dysfunction in TSC is to define to what extent there are cytoarchitectural abnormalities in **non-tuber brain** areas**.** Based on our preliminary data, we propose that there are subtle structural alterations distinct from tubers that may be not be seen by MRI. We have initiated experiments to define P-S6 expression in non-tuber brain areas in 10 post-mortem TSC cases. When completed, our data will represent analysis of the largest post-mortem TSC brain sample to date. These tissues are a precious resource and have been carefully assembled because they share many important phenotypic similarities. All patients had infantile spasms, intractable epilepsy, and significant cognitive disability. Formal IQ testing was not performed but all 10 patients were consigned to institutional living with full management of daily living activities.

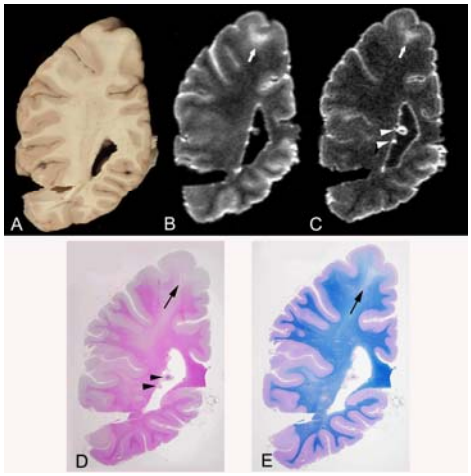

Figure 7. Multiple areas of structural abnormalities (D,E) not detected on post-mortem gross pathology (A) or tissue MRI (B,C).

We have thusfar analyzed P-S6 expression by immunohistochemistry in several non-tuber cortical regions from three post-mortem TSC brain specimens. In these cases post-mortem MRI defined only a fe of the most overt brain lesions. P-S6 immunoreactivity identified numerous regions of aberrant cortical lamination in areas that were histopathologically distinct from tubers. **In these areas, we found 1) small islands of GCs (we term these "microtubers"; Fig.7,8) that express P-S6; 2) only one or two GCs (expressing P-S6) surrounded by multiple P-S6 labeled dysplastic neurons ("dysplasias"; Fig.8,9); or 3) heterotopia (abnormal collections of cells in subcortical white matter).** These data suggest a potentially highly relevant mechanism in which non-tuber lesions may result from enhanced mTOR cascade activation and loss of *TSC1* or *TSC2* in the absence of tuber formation. It is thus possible that other brain areas may contain cells that lack functional *TSC1* or *TSC2* and yet do not form tubers, perhaps due to their embryological origin or progenitor cell subtype.

Increased P-S6 protein labeling serves as a valuable marker for aberrant mTOR activation in cells lacking *TSC1* or *TSC2*. These data raise several pivotal questions:

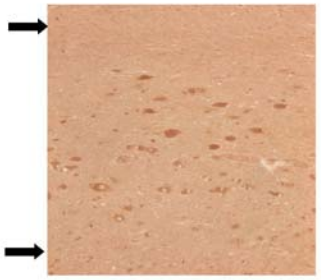

Figure 8. Cluster of P-S6 labeled GCs (microtuber) in subcortical white matter. Arrows depict boundary of overlying normal cortex above, and white mater, below.

1) Does P-S6 expression in these cells result from biallelic gene inactivation? Ongoing analysis in the lab has revealed missense mutations in two non-tuber brain areas consistent with biallelic inactivation as a molecular cause for mTOR activation in these areas.

2) If so, then why are these lesions distinct from tubers? We don't yet understand why some lesions are tubers while others are more subtle structural abnormalities. We are embarking on further genotype analysis to define mutations in other non-tuber brain areas.

3) What are the distinct mechanisms that determine formation of tubers versus more subtle structural abnormalities e.g., loss of TSC1 or TSC2 in specific embryonic brain regions or at specific developmental epochs or in a specific subset of progenitor cell types? Perhaps there are subsets of progenitor cells that are incapable of tuber formation or alternatively, perhaps tubers can form only at precise developmental epochs. Further studies using the methods proposed in this funding initiative are ongoing in my laboratory.

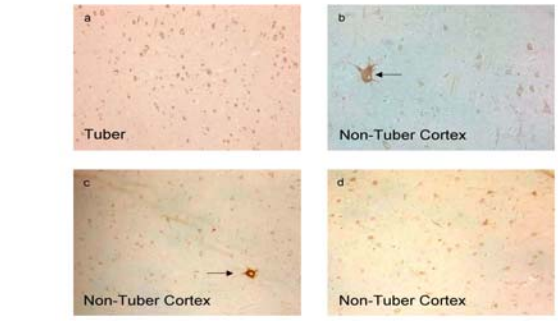

Figure 9. Single P-S6 labeled GC surrounded by smaller P-S6 labeled dysplastic neurons.

4) Are additional pathways (i.e., MAPK, which function in parallel with mTOR activated by loss of TSC1/TSC2), responsible for altered structure? These experiments are in progress in the lab. Previous work from our lab has revealed enhanced MAPK phosphorylation in tubers so a similar mechanism may function in non-tuber brain areas.

5) A recent finding is the detection of P-S6 expression in Purkinje cells in the TSC cerebellum (Fig.9) but not normal control brains. We also find evidence for cytoarchitecutral abnormalities in the cerebellum that have not been previously reported including laminar disarray of the Purkinje cells. This finding is very intriguing since it supports the hypothesis that there is more broad derangement of CNS function in TSC than is evidenced by tuber number. In addition, the cerebellum has been implicated in autism and thus abnormalities of TSC/mTOR signaling in the cerebellum provides a novel avenue for further inquiry.

Figure 10. P-S6 expression in cerebellar Purkinje cells.

Our ongoing work will include a comprehensive analysis of TSC gene mutations in P-S6 labeled cells in non-tuber brain areas as a strategy to define the mutational spectrum of cells in non-tuber brain

areas. We are also in the process of characterizing the localized expression of other kinases within or related to the mTOR cascade that may be aberrantly activated in TSC.

Lineage Markers in TSC Brain Lesions

In a related set of experiments, we focused on defining the embryonic cell origin of giant cells (GCs) and dysplastic neurons (DNs) in brain lesions in TSC since the origin, lineage, and phenotype of GCs and DNs within tubers or SGCTs has not been fully defined. We have previously demonstrated that GCs in cortical tubers and SGCTs exhibit a similar profile of marker proteins as the adult subventricular zone (SVZ), including collapsin response mediator protein-4 (CRMP-4) (Lee et al. 2003). Other studies have found that cortical tubers and SGCTs share common developmental markers including brain lipid binding protein and vimentin (Ess et al. 2005). These findings suggest that tubers and SGCTs may share a common progenitor cell lineage.

Recent evidence suggests that the delta isoform of human glial fibrillary acidic protein (GFAP δ) is specifically expressed in a subpopulation of astrocytes that contain the neural stem cells derived from the human adult subventricular zone. We found that GFAP δ is expressed in cortical tubers, subependymal nodules and in surgically resected samples from 12 TSC patients (Heuer et al., submitted). GFAP δ is expressed in giant cells and dysmorphic astrocytes in tubers as well as diffusely in the cells of SENs and SGCTs. In one large resection specimen, a tuber and SGCT were resected enbloc. Using co-expression of GFAPδand phospho-ribosomal S6 protein we find a contiguous cellular connection between these lesions. GFAPδexpression in tubers, SENs, and SGCTs, suggests that these malformations associated with TSC may derive from a common progenitor cell in the subventricular zone.

Key Research Accomplishments

-comprehensive patho-anatomic analysis of 3 post-mortem TSC brains, with histopathology and post-mortem MRI analysis

-identification of phospho-S6 labeled cells in brain areas distinct from tubers in post-mortem TSC brain tissue

-identification of subtle dysplasias and morphological abnroamities in non-tuber brain regions including subcortical areas

-identification of P-S6 expression in Purkinje cells in the cerebellum suggesting enhanced mTOR signaling

-identification of isolated giant cells in non-tuber brain areas

-optimiziation of single cell DNA sequence analysis

-identification of missense second hit mutations in non-tuber brain areas

-identification of additional marker proteins (GFAP delta isoform) for giant cells in TSC

Reportable Outcomes

L Marcotte. PB Crino, Structural Abnormalities in Non-Tuber Cortex in Tuberous Sclerosis Complex (TSC), American Academy of Neurology Meeting 2007, Boston, MA

Crino PB. Molecular pathogensis of tuber formation in TSC. International TSC research Conference, Annapolis, MD, Plenary Talk

K. Boer, S. Redeker, F. Jansen, M. Nellist, A. M. W. van den Ouweland, J.J.G. Geurts, J.A. Castelijns, W.G.M. Spliet, D. Troost, P. Crino and E. Aronica. Tuberous sclerosis complex: a clinicopathological and immunohistochemical study of an autopsy case, submitted.

G. Heuer, E. M. Hol, M. Frost, E. Aronica, L Marcotte, P.B. Crino. Expression of GFAP δ in tubers, subependymal nodules, and subependymal giant cell tumors in tuberous sclerosis complex, submitted

J Yu, M Baybis, G Baltuch, E Uhlmann DH Gutmann, KL Nathanson, Biallelic *TSC1* gene inactivation in Tuberous Sclerosis Complex giant cells, in preparation

Conclusions –"So what?"

These data provide pivotal new insights into the pathological spectrum of disease in TSC. The identification of subtle cytoarchitectural abnormalties not detected by MRI yields clues as to why many individuals with TSC suffer from severe epilepsy or autism even when the MRI scan reveals only a solitary tuber or brain lesion. These data suggest that for many TSC patients structural lesions in the brain are widespread and pervasive and further demonstrate the severe consequences of TSC gene mutations on neurological functioning. Our data when completed, will also provide a compelling clinical case for early and in fact possibly in utero treatment with mTOR inhibitors such as rapamycin to prevent the effects of TSC gene mutations on brain formation. The identification of cerebellar pathology in TSC provides a new previously unexplored brain area in which structural changes may have significant neurological sequellae. Upon completion of our proposed stufies we wil have defined for the first time a comprehensive molecular-anatomic view of a neurodevelopmental disorder associated with epilepsy and autism. Finally, id4ntificaqtion of new marker proteins for brain lesion in TSC provides new insights into how brain lesions form during pre- and post-natal development and again, yield clues to possible therapeutic interventions for TSC.

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